

Specificity and Sensitivity of Radioimmunoassay for Hepatitis B Antigen

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Sera from a survey of 6,026 people were tested for hepatitis B surface antigen by using radioimmunoassay and counterelectrophoresis. Forty-eight sera (0.79%) were positive by counterelectrophoresis and 152 sera (2.52%) were positive by radioimmunoassay, using the most liberal of the recommended criteria for positivity (i.e., counts 3 standard deviations above the mean). Absorption tests performed on the 152 radioimmunoassay-positive sera showed that 10 (6.6%) were false-positive reactions to guinea pig protein, 74 (48.6%) were due to false-positive reaction(s) with other protein(s) in the test system, and 68 (44.8%) were true positives. There was a strong correlation between the degree of elevation of radioactive counts and the proportions of sera that were true positives; all 49 sera with counts >50 standard deviation units above the mean were true positives, but only 19 (18.4%) of the 103 sera with counts <50 standard deviation units were true positives. A few sera with high counts required absorption with type-specific (type D) antisera. The following conclusions were reached from this study: (i) absorption tests should be run on all radioimmunoassay-positive, counterelectrophoresis-negative sera; (ii) most (about 90%) false positives are not due to anti-guinea-pig protein reactions; and (iii) radioimmunoassay, in combination with absorption tests, yields a modest increase (about 35%) in detection of true positives over use of counterelectrophoresis alone.

Solid-phase radioimmunoassay (RIA) is one of the most sensitive and convenient techniques for detecting hepatitis B antigen (HB_sAg) in serum (4). An unresolved problem in the use of this technique is the lack of consensus on the level of radioactivity in counts per minute that constitutes a positive test. A further difficulty has been reported recently by Sgouris (9), Prince (8), and Alter (2), who have demonstrated that many sera that are positive by RIA but negative by counterelectrophoresis (CEP) are false-positive reactions due to nonspecific human antibody against guinea pig proteins used in the test system. Resolution of these problems is required to avoid misclassification of sera tested by RIA. This survey reports efforts to determine the sensitivity and specificity of RIA, by using the Ausria kit (Abbott Laboratories, N. Chicago, Ill.), for HB_sAg in the largest collection of sera reported.

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MATERIALS AND METHODS

Source of sera. The sera used in this study were obtained during a serologic survey for HB_sAg, which was conducted at Fort Hood, Texas during February and March of 1973. The survey was part of an investigation of an outbreak of viral hepatitis in soldiers (A. M. Allen and G. R. Irwin, unpublished data). Sera were collected from 6,026 military and civilian personnel with no signs or symptoms of hepatitis. In addition, sera were obtained from 85 patients with acute hepatitis.

HB_sAg tests. The solid-phase radioimmune assay for HB_sAg (Ausria kit from Abbott Laboratories, N. Chicago, Ill.) was performed as described by Ling and Overby (7). The Ausria kits used were those commercially available from June 1972 through May of 1973. These kits did not contain cold guinea pig serum in the ¹²⁵I-labeled guinea pig anti-hepatitis B_s antigen. Briefly, 0.1 ml of serum to be tested was placed in plastic tubes coated with guinea pig anti-hepatitis B_s (anti-HB_s) and incubated for 16 h at room temperature. Each tube was washed with five 2-ml samples of tris(hydroxymethyl)aminomethane buffer. After thorough aspiration of the remaining buffer from the bottom of the tube, 0.1 ml of ¹²⁵I-labeled guinea pig

anti-HB_s was added and incubated for 90 min at room temperature, and the tubes were washed as described above and counted in a model 1185 Searle (Nuclear-Chicago Corp., Des Plaines, Ill.) gamma counter. The manufacturer's negative control serum was dispensed 10 times into separate tubes and processed as above for all runs. All CEP-positive sera were checked for HB_sAg specificity by immunodiffusion against rabbit anti-HB_s and standard HB_sAg. CEP-positive sera were considered HB_sAg-positive if lines of identity with standard HB_sAg were obtained.

Absorption tests for HB_sAg specificity. Sera positive by RIA (i.e., count per minute 3 standard deviations [SD] above the mean of 10 replicates of the negative control; or according to the Abbott criteria, 2.1 times the mean of negative control sera) and negative by CEP were analyzed for specificity to HB_sAg by means of the following absorption test. Samples of 0.1 ml of the serum to be tested were incubated at room temperature for 2 h with 0.025 ml of each of four absorption sera, normal guinea pig serum, guinea pig anti-HB_s, normal rabbit serum, and rabbit anti-HB_s. In addition, duplicate samples were run without absorption. After incubation, a 0.1-ml amount of the absorbed serum was pipetted into Aursia tubes and processed as above. Since rabbit and guinea pig anti-HB_s had been shown to decrease radioactive counts of CEP-positive sera by 80% or more, RIA-positive and CEP-negative sera showing a decrease in counts per minute in excess of 80% with rabbit or guinea pig anti-HB_s (but not by normal rabbit or guinea pig sera) were considered specific for HB_sAg.

RESULTS

Sensitivity. The sera obtained from 6,026 asymptomatic subjects at Fort Hood were tested for HB_sAg by RIA and CEP (Table 1). According to one's choice of three recommended criteria for RIA-test positive (5, 7), RIA was 2.2, 2.5, or 3.2 times more sensitive than CEP (positive RIA test: 2.1 times the mean, 5 SD above the mean, or 3 SD, respectively). The manufacturer's criteria of 2.1 times the mean ranged between 4.4 and 13.6 SD units on 38 runs and averaged 8.3 SD units.)

HB_sAg specificity. The 152 sera which were positive by RIA were tested for specific reactivity for HB_sAg. Each of the 48 sera which were positive by CEP (as well as by RIA) gave precipitin lines of identity with HB_sAg standards used for subtyping with rabbit antisera.

Absorption tests were performed to determine the specificity of RIA-positive, CEP-negative sera for HB_sAg. The sera were classified as true or false positives according to the following criteria: (i) true positive sera, sera showing an 80% or more decrease in counts per minute after absorption with guinea pig or rabbit anti-HB_s, but not with normal animal

sera, (ii) false-positive anti-guinea pig protein, sera blocked by normal guinea pig serum; and (iii) indeterminate false-positive, sera inhibited by neither specific anti-HB_s nor by normal animal sera. The latter form of false positive was labeled indeterminate because its mechanism is unknown, but the former was referred to as anti-guinea pig protein to indicate nonspecific reactivity to guinea pig protein used in the test. Examples of the results obtained after absorption testing are shown together with their interpretation in Table 2.

With the criteria outlined above, 68 (44.8%) of the 152 RIA-positive sera were classified as true positive, 10 (6.6%) were false positives (anti-guinea pig protein), and 74 (48.6%) were indeterminate false positives. Fifty-five (74%) of the sera classified as indeterminate false positives had radioactivity counts between 3 SD units above the mean and 2.1 times the mean of the negative control. The counts in this range were too low to yield conclusive results with absorption tests. Fourfold concentration of six of these sera failed to resolve the problem, primarily because the counts did not show a proportionate increase with concentration.

Four RIA-positive, CEP-negative sera with radioactivity counts greater than 20 SD units above the negative control mean were not inhibited by animal sera containing anti-HB_s specific for subtype Y. Repeat testing with sera containing anti-HB_s against subtype D showed significant inhibition. Unabsorbed serum showed 5,554 counts/min, and normal guinea pig serum showed 5,864 counts/min. Guinea pig HB_sAb serum specific for subtype Y showed 3,174 counts/min, and the guinea pig HB_sAb serum specific for subtype D showed 630 counts/min.

There was a correlation between the degree of

TABLE 1. *Hepatitis B antigen positivity in sera from 6,026 asymptomatic people testing with RIA and CEP*

Test results ^a		Subjects	
CEP	RIA	No.	%
Positive	Counts/min > 3 SD ^b	48	0.79
Negative	Counts/min > 2.1 × m ^c	56	0.93
Negative	2.1 × m > counts/min	15	0.25
	5 SD		
Negative	5 SD > counts/min	33	0.55
	3 SD		

^a Based on radioactivity in counts per minute.

^b SD, Standard deviations above the mean of the negative control.

^c m, Mean of the negative control.

TABLE 2. Absorption of RIA-positive sera to determine specificity for hepatitis B antigen/representative test results in counts per minute (employing Ausria kits)

Serum no.	Unabsorbed sera		Absorbed sera				Interpretation
	CEP	RIA	Normal guinea pig serum	Guinea pig HB _e Ab	Normal rabbit serum	Rabbit HB _e Ab	
032	Positive	10,051	8,352	826	8,600	852	True positive
F 80	Negative	7,263	7,650	1,049	5,430	287	True positive
F 1226	Negative	1,372	442	452	1,329	1,388	False positive (anti-guinea pig protein)
F 1769	Negative	1,883	472	399	1,295	838	False positive (anti-guinea pig protein)
M 744	Negative	739	775	794	863	728	Indeterminate false positive
M 1895	Negative	518	383	428	646	594	Indeterminate false positive
Positive control (rabbit anti-guinea pig protein)		16,175	511	492	13,461	16,256	False positive (anti-guinea pig protein)

elevation of radioactivity counts found on RIA testing and the proportion of tests classified as true or false positives after absorption tests (Table 3). The higher the counts in SD units above the mean of the negative control, the more likely were the sera to be truly positive. All of the 49 sera with counts above 50 SD units were classified as true positives, while only 19 (18.4%) of the 103 sera with counts between 3 and 50 SD units were so classified. Seventy-two percent of the true positive sera had counts per minute in excess of 50 SD units.

Sensitivity and specificity in patients versus asymptomatics. The sensitivity and specificity of the RIA test for HB_eAg in sera obtained from asymptomatic people was compared to that in sera obtained from patients with acute hepatitis (Table 4). As compared to the results of CEP testing, RIA appeared to be relatively more sensitive in asymptomatic individuals than in patients; however, the difference was not statistically significant ($\chi^2 = 0.5$, $P > .25$), and the average increase in sensitivity over CEP for patients as well as asymptomatics was 27%. No nonspecific reactions for HB_eAg occurred in patients.

DISCUSSION

The solid-phase RIA is reported to be up to 500 times more sensitive than CEP for detecting HB_eAg in serial dilutions of known positive sera (4). Used as a screening test in blood donors, RIA has detected as many as 10 times the number of positives detected by CEP (8). Recently, most of this apparent difference in sensitivity has been found to be due to nonspe-

TABLE 3. Frequency distribution of absorption-tested sera according to the counts obtained in RIA for hepatitis B antigen

Counts (SD above mean)	True positives	False positives	
		Anti-guinea pig protein	Indeterminate
3-	2 (2.9) ^a	0 (0)	31 (41.9)
5-	6 (8.8)	2 (20.0)	24 (32.4)
10-	1 (1.5)	6 (60.0)	11 (14.9)
20-	10 (14.7)	2 (20.0)	8 (10.8)
50-	27 (39.7)	0 (0)	0 (0)
100-	12 (17.6)	0 (0)	0 (0)
200-	10 (14.7)	0 (0)	0 (0)

^a Figures in parentheses represent percentages of total. Positivity criterion of the manufacturer (i.e., 2.1 times the mean) ranged between 4.4 and 13.6 SD units on 38 runs, and averaged 8.3 SD units.

cific reactions to guinea pig and other proteins in the test system (1, 10), and revised estimates of sensitivity now indicate that RIA will detect only two to three times the number of true positives as CEP. Our results suggest that the actual difference in sensitivity is much more modest, being only on the order of 25% greater than CEP. Whether the discrepancy between our results and those of others is due to differences in technique or in the populations sampled remains to be determined.

Abbott Laboratories has recently modified the RIA kit by adding guinea pig serum to the radioactive labeled anti-HB_e (6). It is hoped that by this step the problem with nonspecific reactivity to guinea pig protein will be overcome. Although we have limited experience

TABLE 4. Sensitivity and specificity of RIA for hepatitis B antigen in sera from 85 hepatitis patients and from 6,026 asymptomatic subjects

Subjects	True positives ^a			False positives	
	RIA (+) and CEP (+)	RIA (+) and CEP (-)	Totals	Anti-guinea pig protein	Indeterminate
Patients	30	8	38	0	0
Asymptomatics	48	20	68	10	74

^a RIA (+), RIA-positive; CEP (+), CEP-positive; CEP (-), CEP-negative.

with the new test system, the data in this report suggest that its use would have resulted in only a 10% decrease in the number of false positives detected by RIA.

The majority of false-positive reactions appear to be what we have called indeterminate false positives. The exact cause of this reaction is unknown, but it is presumably due to other serum components within the test system. In this survey, none of 49 asymptomatic subjects whose sera were initially classified as indeterminate false positive had abnormal test results when rebled 3 months later. In contrast, each of seven subjects who were classified as anti-guinea pig false positive was RIA positive 3 months later, and 28 (90%) of 31 true positive asymptomatics were again RIA positive after 3 months. These findings indicate that the factors responsible for indeterminate false-positive tests were transient in their occurrence and may be due to temporally associated phenomena in the host or in the test system. A new RIA test from Abbott Laboratories, Ausria II, appears to significantly decrease this second type of false-positive reaction by altering time and temperature of incubation from 16 h at room temperature to 2 h at 45 C (personal communication). In addition the ¹²⁵I-labeled anti-HB_s is of human origin. The Ausria II test appears to be an improved RIA test for detecting HB_sAg, particularly for blood banks.

Absorption tests may occasionally show that sera which are strongly positive by RIA but negative by CEP cannot be inhibited either by normal guinea pig serum or by guinea pig anti-HB_s. In this study and in at least one other (4), the cause was found to lie in subtype specificity of the anti-HB_s used in the inhibition tests. Sera containing subtype Y may be missed unless subtype-specific antisera are used; most commercial antisera are usually satisfactory for subtype D (6). RIA-positive and CEP-negative

sera with relatively low radioactivity counts (counts/min $2.1 \times$ mean) are difficult to subtype by absorption-inhibition testing because of weak reactivity. However, when inhibition of sera with elevated counts per minute can be obtained by anti-HB_s-differing specificities (anti-ayw versus anti-adw), it is possible to confirm not only the presence of HB_sAg, but to suggest that the individual serum was of the subtype inhibited by the appropriate anti-HB_s. Theoretically, it may be possible to distinguish HB_sAg determinants W and R by this method (3).

For research purposes, our laboratory used 3 SD units above the mean on two different occasions as the criterion of a positive RIA test. Sera that were negative by CEP then underwent absorption tests for specificity. However, the results of specificity testing can largely be inferred from the RIA-test result itself (Table 3): high radioactivity counts (>50 SD above the mean) provide reasonable assurance that the serum is a true positive, but low counts (<20 SD above the mean) indicate the strong probability that the serum is a false positive.

With serially diluted CEP-positive sera, HB_sAg can be routinely detected to the level of 10^{-4} or 10^{-5} by using the criterion of 3 SD above the mean to indicate of HB_sAg. However, an individual serum obtained from a clinical source having counts per minute equal to points on a standard HB_sAg titration curve cannot be assumed to contain HB_sAg without specificity testing.

The need for further evaluation refinement of the RIA test for HB_sAg is obvious. However, it remains the most sensitive of the available tests, and the problems of specificity can be overcome by means of appropriate procedures.

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